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Myoblast-mediated gene transfer for therapeutic angiogenesis and arteriogenesis

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Therapeutic angiogenesis aims at generating new blood vessels by delivering growth factors such as VEGF and FGF. Clinical trials are underway in patients with peripheral vascular and coronary heart disease. However, increasing evidence indicates that the new vasculature needs to be stabilized to avoid deleterious effects such as edema and hemangioma formation. Moreover, a major challenge is to induce new vessels that persist following cessation of the angiogenic stimulus. Mature vessels may be generated by modulating timing and dosage of growth factor expression, or by combination of 'growth' factors with 'maturation' factors like PDGF-BB, angiopoietin-1 or TGF-β. Myoblast-mediated gene transfer has unique characteristics that make it a useful tool for studying promising novel approaches to therapeutic angiogenesis. It affords robust and long-lasting expression, and can be considered as a relatively rapid form of 'adult transgenesis' in muscle. The combined insertion of different gene constructs into single myoblasts and their progeny allows the simultaneous expression of different 'growth' and 'maturation' factors within the same cell *in vivo*. The additional insertion of a reporter gene makes it possible to analyze the phenotype of the vessels surrounding the transgenic muscle fibers into which the myoblasts have fused. The effects of timing and duration of gene expression can be studied by using tetracycline-inducible constructs, and dosage effects by selecting subpopulations consistently expressing distinct levels of growth factors. Finally, the autologous cell-based approach using transduced myoblasts could be an alternative gene delivery system for therapeutic angiogenesis in patients, avoiding the toxicities seen with some viral vectors.
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Abbreviations: FACS, fluorescence-activated cell sorting; FGF, fibroblast growth factor; flk-1, fetal liver kinase 1(= VEGFR-2); flt-1, fms-like tyrosine kinase 1(= VEGFR-1); PDGF, platelet-derived growth factor; HIF-1α, hypoxia-inducible factor 1 alpha; PECAM, platelet endothelial cell adhesion molecule; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

Therapeutic angiogenesis from bench to bedside

Therapeutic angiogenesis holds promise as a medical treatment for patients with coronary and peripheral artery disease (Yla-Herttuala & Alitalo, 2003). Numerous studies using animal models have provided evidence for the efficacy of this approach. A variety of angiogenic growth factors, delivered as recombinant proteins or genes using different vectors and application methods, have been shown to induce angiogenesis and collateral artery growth, leading to improved regional blood flow and tissue salvage (Isner, 2002; Abo-Auda & Benza, 2003). So far, however, the translation of these encouraging preclinical findings to patients has yielded mixed results (Khurana & Simons, 2003; Yla-Herttuala & Alitalo, 2003). Primary clinical end points such as exercise tolerance testing or quality-of-life measurement were missed in several placebo-controlled studies of therapeutic angiogenesis, whereas other studies showed clinical improvements (Lederman *et al*., 2002a, b; Simons *et al*., 2002; Henry *et al*., 2003; Khurana & Simons, 2003; Yla-Herttuala & Alitalo, 2003). However, many findings are preliminary as the studies were conducted in rather small patient populations and some were designed as safety and feasibility studies using low doses of gene vectors (for a recent review, see Yla-Herttuala & Alitalo, 2003). The question of whether therapeutic angiogenesis has the potential to provide clinical benefit to patients, therefore, still awaits clarification through ongoing larger-scale phase III trials.

The 'first generation' of clinical trials to promote angiogenesis primarily focused on inducing capillary and collateral growth by overexpression of a single angiogenic growth factor (Isner, 2002). Given the as yet inconclusive results of the first clinical angiogenesis trials described above, delivery of one factor alone may not suffice to achieve robust therapeutic effects, and further research seems warranted. Increased knowledge of the involvement and crosstalk of different cell types, growth factors and matrix components could lead to the development of strategies aimed at stimulating angiogenesis in a more controlled way (Blau & Banfi, 2001; Jain, 2003). Vessel maturation and remodeling may need to be stimulated in parallel with the induction of angiogenesis in order to produce functional and stable vessels. The present review describes the

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method of myoblast-mediated transfer of angiogenic genes, and discusses its role as a tool for a multifactorial approach to therapeutic angiogenesis. This cell-based approach, using engineered autologous muscle precursor cells, has unique advantages as a relatively rapid and inexpensive method of 'adult transgenesis'. Primary myoblasts can be genetically engineered and characterized *in vitro*. Upon intramuscular injection, they become incorporated into resident adult muscle fibers. This allows the study of combinations of angiogenic growth factors, different expression periods and doses in a systematic way using the same gene delivery system. Importantly, the gene of interest is expressed by the skeletal muscle fiber itself, the main target tissue of therapeutic angiogenesis for peripheral artery disease.

Maturation and stabilization: the 'well-tempered vessel'

Vessels induced by overexpression of a single angiogenic growth factor have been described to lack morphological, architectural and functional characteristics of mature capillaries (Epstein *et al*., 2001). The new vessels induced by VEGF, for example, are often leaky, irregular and unstable, and can develop into hemangiomas (Springer *et al*., 1998; Lee *et al*., 2000; Sundberg *et al*., 2001; Dvorak, 2002). A number of VEGF-induced effects synergize to destabilize the newly formed blood vessels, which is necessary for the angiogenic response, but may lead to uncontrolled and irregular vessel growth. VEGF is a strong inducer of endothelial cell proliferation and migration, effects that are mediated through the combined binding of VEGF to the receptors flk-1 (VEGFR-2) and flt-1 (VEGFR-1) expressed on endothelial cells (Neufeld *et al*., 1999; Autiero *et al*., 2003). In addition, a positive feedback loop may be created by the chemotactic effects of VEGF on monocytes/macrophages, which in turn release more VEGF and other angiogenic growth factors (Barleon *et al*., 1996; Neufeld *et al*., 1999; Clauss *et al*., 1990). Furthermore, the activity of proteases (such as matrix metalloproteinases) can be increased, which contribute to degrading the capillary basement membrane and extracellular matrix, and have been shown to reactivate the angiogenic activity of VEGF bound there (Zucker *et al*., 1998; Hashimoto *et al*., 2002; Bergers *et al*., 2000). The vessels are further destabilized by the induction of angiopoietin-2, which can lead to a disruption of the communication between the endothelial cells and the surrounding mesenchyme (Maisonpierre *et al*., 1997; Mandriota & Pepper, 1998). Taken together, VEGF actions can lead to the desired induction of new vessels through intussusception, bridging and sprouting. However, the effects of VEGF can be deleterious if uncontrolled (Carmeliet, 2000; Blau & Banfi, 2001; Epstein *et al*., 2001). Increased leakiness has been reported to lead to severe edema (Luttun *et al*., 2002), and has been linked to limb loss in several animal experiments (Masaki *et al*., 2002; Rissanen *et al*., 2003). The appearance of glomeruloid vascular proliferation has been reported in different tissues and using different gene vectors (Schwarz *et al*., 2000; Sundberg *et al*., 2001; Dor *et al*., 2002). When VEGF is delivered long-term, vascular proliferation may be unabated, often resulting in hemangioma growth (Springer *et al*., 1998; Lee *et al*., 2000). On the other hand, vessels induced by short-term VEGF expression have been found to be unstable and to regress promptly following cessation of the angiogenic stimulus (Dor *et al*., 2002). Thus, a growing body of evidence suggests that the induction of newly formed vessels must be followed by their maturation. Cell types, growth factors and matrix components need to be balanced in order to give rise to vessels that are stable, conductive and capable of regulating their permeability in a physiological way (Blau & Banfi, 2001; Jain, 2003). Various strategies to stimulate vessel maturation in angiogenesis have been proposed, which can be classified into three broad categories. (1) Controlling the timing, duration and dosage of VEGF expression could avoid deleterious effects while leading to stable and mature vessels. This hypothesis is based, in part, on observations of a transgenic mouse model of conditional VEGF expression, in which less than 2 weeks of VEGF expression was found to generate immature vessels that regressed following withdrawal of the angiogenic stimulus, whereas longer-term VEGF expression led to stable and persistent vessels (Dor *et al*., 2002). In comparison with transgenic approaches, myoblast-mediated gene transfer is a more readily flexible system that allows a systematic investigation of the dosage and timing of any given growth factor in skeletal muscle. (2) Codelivery of 'maturation' factors regulating the interplay between endothelial cells and pericytes, such as PDGF-BB, TGF-β and angiopoietins, together with the primary angiogenesis-inducing growth factor VEGF could modulate or balance VEGF-induced effects (Richardson *et al*., 2001). This hypothesis is based on the observation that the recruitment of pericytes stabilizes capillaries, regulates their permeability and guides branching during angiogenesis (Nehls *et al*., 1992; 1994; Benjamin *et al*., 1998; 1999; Morikawa *et al*., 2002). Recently, the combined delivery of FGF-2, another angiogenic growth factor, and the 'maturation factor' PDGF-BB was shown to lead to more mature vessels compared to FGF-2 alone (Cao *et al*., 2003). In addition, the blocking of PDGF-BB signaling was found to delay tumor growth (Bergers *et al*., 2003). However, the mechanisms involved in the recruitment and physiological function of pericytes are not yet fully understood. (3) The overexpression of transcription factors upstream of the endogenous angiogenic cascade of events, such as HIF-1α, may lead to a more pleiotropic and orchestrated response involving different factors and cell types (Elson *et al*., 2001). Alternatively, angiogenic genes may be introduced in such a way that they are expressed and spliced in a manner that better reflects the intrinsic transcriptional profile of the target tissue, such as the introduction of unspliced genomic VEGF constructs (R. Crystal, oral communication) or the upregulation of the endogenous VEGF gene (Rebar *et al*., 2002). Clearly, these approaches appear very promising in achieving the goal of inducing mature, functional and stable vessels by gene transfer. The systematic analysis of timing, dosage and combination of angiogenic 'induction' and 'maturation' factors should make it possible to test these parameters rigorously. Such studies should provide a stronger fundamental basis for the next generation of clinical trials of therapeutic angiogenesis.

Myoblast-mediated gene transfer

A brief summary of the procedure of isolation, culture, retroviral transduction and reimplantation of primary myo-

blasts is given here (for a detailed description, see Banfi *et al*., 2002). Skeletal muscle is harvested from neonatal mice, minced and digested in collagenase and dispase. The dissociated cells obtained are then plated in culture on collagen-coated dishes in media supplemented with fetal bovine serum and human FGF-2. Myoblasts are separated from contaminating cells such as fibroblasts by taking advantage of their differential adherence to the dish over several passages. Alternatively, myoblasts can be separated from contaminating cells by flow-sorting or magnetic bead separation based on their specific expression of alpha 7 integrin (Blanco-Bose *et al*., 2001). Finally, purity of the primary population is confirmed by morphological criteria and desmin staining. The ability to differentiate can be tested *in vitro* by growing the myoblasts in growth factor-deprived medium. After several days to 1 week, differentiation is readily apparent by the formation of elongated multinucleated myotubes, which start to twitch randomly. For transduction with the gene of interest, high-titer retroviral supernatants are obtained from the helper-free, ecotropic ΦNX packaging cell line (Pear *et al*., 1993). Myoblasts are transduced by four sequential rounds of infection, typically resulting in a transduction efficiency of 99% (Springer & Blau, 1997). Myoblasts can then be injected into the muscle at a concentration of 10^5 cells μl^{-1}, where they either fuse with each other to form myotubes or fuse with pre-existing muscle fibers (Figure 1).

Myoblast-mediated gene transfer using retrovirally transduced myoblasts differs from other gene delivery systems in important ways. The gene of interest is expressed over a long period of time, assuming the use of appropriate promoters. Indeed, the transgene has been found to be expressed for at least 10 months, a significant portion of the average lifespan of a mouse (Wang *et al*., 1997). Large and multiple gene products can be inserted. A single gene insert can be up to 6 kb in size approximately, a limit set by the capacity of the retroviral vectors used for *ex vivo* transduction of the myoblasts. Subsequently, additional genes can be introduced by reinfecting the cells with retroviral constructs carrying other genes of interest. The high capacity of this gene delivery system in comparison with direct viral vector administration is exemplified by myoblasts that have been engineered in our laboratory, which simultaneously expressed four different genes under the control of different promoters. Significantly, the myoblasts retained their capacity to differentiate *in vitro* and to fuse *in vivo* following these multiple rounds of infection (unpublished data). As the retroviral transduction of the myoblasts is carried out *ex vivo* in the culture dish, the muscle tissue is not directly exposed to infectious virus, avoiding possible complications resulting from immune responses in the area of interest (St George, 2003). Furthermore, using myoblasts engineered *ex vivo*, the expression of the gene product can be measured in the culture dish before implantation and normalized to the number of cells, making it a useful tool for the investigation of dose-dependent effects of angiogenic growth factors on vessel phenotypes. Since the injected myoblasts fuse with pre-existing myofibers or fuse with each other to form myotubes,

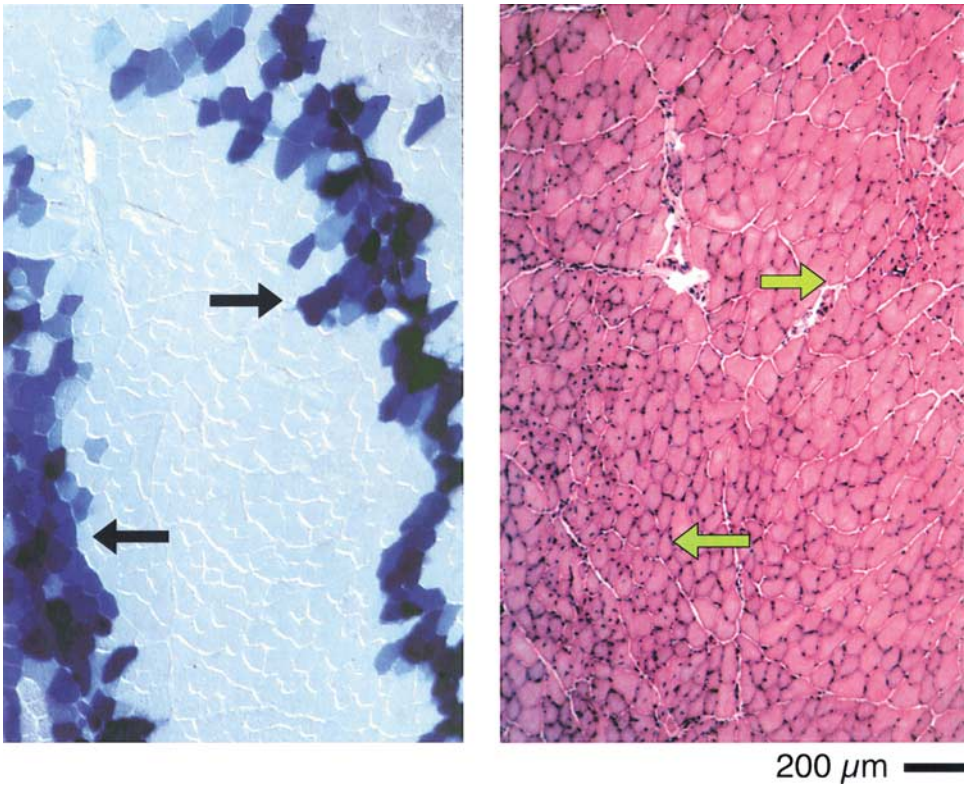

Figure 1 Following injection into the muscle, transduced myoblasts expressing the beta-galactosidase reporter gene either fuse with each other to form myotubes or fuse with pre-existing muscle fibers. Hematoxylin/eosin staining (right panel) shows that the tissue architecture is fully preserved, the area of engraftment being recognizable only by the centralization of the muscle nuclei (yellow arrows). Note the absence of inflammatory cells. Enzymatic beta-galactosidase staining on a neighboring section (left panel) shows the expression of the reporter gene by the transgenic muscle fibers (blue arrows). With permission from Dekker Publishing group: Ozawa *et al*. (2000) in Templeton & Lasic eds.: Gene Therapy: therapeutic mechanisms and strategies.

the angiogenic genes are only expressed in a defined area of the target tissue itself, skeletal muscle (Figure 1).

Transduced myoblasts as a gene delivery system for therapeutic angiogenesis

The high and long-term expression of VEGF afforded by myoblast-mediated gene transfer has provided new insights into the pathophysiology and possible deleterious effects of this growth factor. Studies using this delivery method have led to the discovery of the potential of VEGF to induce the growth of hemangioma-like structures (Springer *et al*., 1998; Lee *et al*., 2000). Subsequently, the growth of aberrant glomeruloid vessels has been observed using other gene delivery systems and in different tissues, confirming the results of the myoblast-mediated delivery (Schwarz *et al*., 2000; Sundberg *et al*., 2001; Dor *et al*., 2002). The long-term overexpression of angiogenic growth factors afforded by transduced myoblasts makes it possible to modulate the duration and levels of transgene expression and to study the effects on the phenotype of induced vessels. At least two different strategies can be pursued. A tetracycline-inducible system has been developed, which makes it possible to interrupt the expression of the gene of interest after a given period, or to switch it on at a desired time point (Kringstein *et al*., 1998; Blau & Rossi, 1999). This system could be used to define basic parameters such as the time windows necessary for the successful induction of specific effects *in vivo*, stimulation of vessel growth, maturation and remodeling or induction of deleterious effects. The levels of gene expression could be modulated by isolating subpopulations of a heterogeneous population of transduced primary myoblasts by FACS sorting. Using this strategy, a systematic study of the phenotypes of new vessels induced by different levels of growth factors is possible (Ozawa & Banfi *et al*., submitted). The combination of both approaches should make it possible to study the effects of timing and dosage of single and combined angiogenic growth factors simultaneously or sequentially in the same animal using the same delivery system.

Recent results indicate that by differential expression of VEGF alone, very different vessel phenotypes can be obtained. The formation of hemangioma-like structures has been described above (Figure 2). More recently, it was found that in the areas adjacent to the implantation site of the VEGF-producing myoblasts, the typical pattern of the capillary network in skeletal muscle was replaced by numerous smooth-muscle-cell-coated vessels (Figures 3 and 4) (Springer *et al*., 2003). Similar phenotypes were found in areas of reduced myoblast engraftment. Based on their cellular composition and architecture, these vessels were classified as arterioles. It is presently not known if arteriole formation was due to a specific level of VEGF expression or a shear-stress-dependent mechanism that would occur in the vicinity of an area of active blood vessel growth. In other areas, increased vessel densities were observed composed of capillaries with more physiological morphology and architecture (Figure 3). These patterns closely resemble those described by other investigators in therapeutic angiogenesis studies using different gene vectors and animal models. Other groups have shown that myoblast-mediated gene transfer of VEGF can lead to functional angiogenesis with increased blood flow without hemangioma formation. Recently, VEGF-expressing myoblasts transduced with the hemagglutinating virus of Japan were implanted in the damaged hearts of rats, leading to new capillaries of physiological appearance (Suzuki *et al*., 2001). Significantly, no hemangioma formation was seen in this study in which the expression of VEGF was transient. Similar findings were made using bioartificial muscle-mediated gene

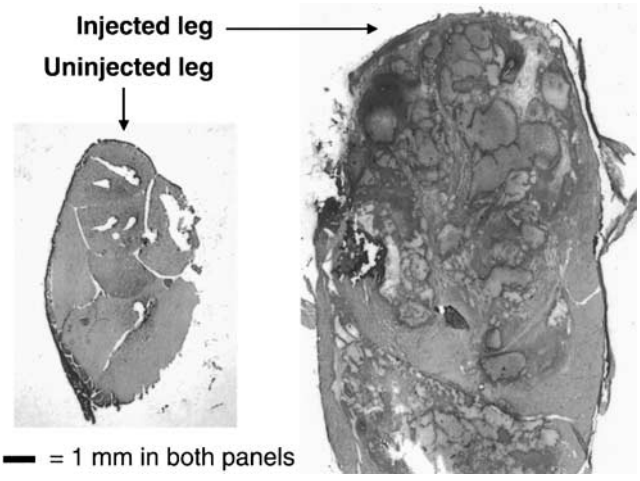

Figure 2 Large vascular structures with blood pools develop in skeletal muscle implanted with primary myoblasts expressing VEGF. Tissue was harvested 44–47 days following myoblast implantation (hematoxylin/eosin staining). The control leg shows normal size and morphology (left panel), whereas the leg injected with VEGF-expressing myoblasts shows hemangioma leading to a more than two-fold increase in leg size (right panel). With permission from Elsevier Science: Springer *et al*. (1998). VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol. Cell*, 2, 549-558.

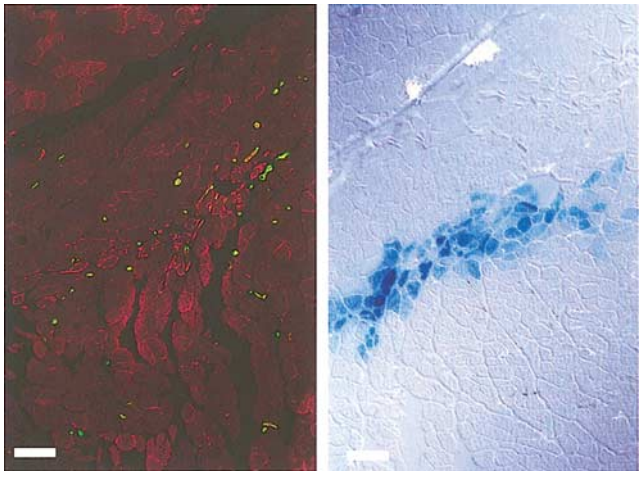

Figure 3 Tibialis anterior muscle injected with VEGF-expressing myoblasts: a few myoblasts have leaked back through the wound created by the needle during injection and fused with muscle fibers along the track as shown by the beta-galactosidase expression (blue fibers in the right panel). No hemangioma formation is seen, but areas of higher capillary density of normal morphology are apparent. The left panel shows immunofluorescence for PECAM/CD31 (red) and alpha-smooth-muscle actin (green). Around these areas, numerous arterioles have formed, which replace the capillary pattern typically present in skeletal muscle. Bar 100 μm. With permission from Elsevier Science: Springer *et al*. (2003). Localized arteriole formation directly adjacent to the site of VEGF-induced angiogenesis in muscle. *Mol. Ther*., 7, 441-449.

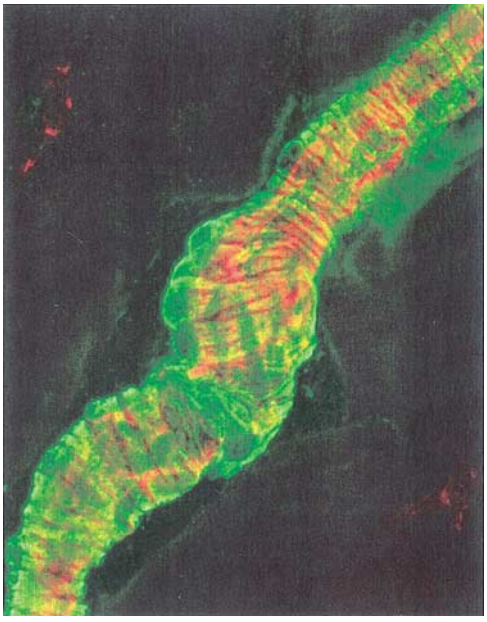

Figure 4 Confocal microscope generated three-dimensional reconstruction of an arteriole near an implantation site (immunofluorescence for the endothelial cell marker PECAM/CD31 (red) and alpha-smooth-muscle actin (green)). Green-stained smooth-muscle cells are seen wrapping around a red-stained endothelial tube. Bar 10 μm. With permission from Elsevier Science: Springer *et al*. (2003). Localized arteriole formation directly adjacent to the site of VEGF - induced angiogenesis in muscle. *Mol. Ther*., 7, 441-449.

transfer of VEGF, in which retrovirally transduced and terminally differentiated myoblasts bioengineered following a special protocol were implanted into the leg and heart (Lu *et al*., 2001; 2002).

In summary, these findings provide support for the concept that the phenotypes of new vessels induced by gene transfer are dictated by the levels or duration of VEGF expression. The time window and the dosage necessary to induce angiogenesis, vessel maturation and hemangioma formation are currently being addressed using the regulatable constructs and subpopulations isolated by FACS.

In clinical trials, the implantation of primary myoblasts for therapeutic purposes into the leg and heart has been shown to be feasible (Gussoni *et al*., 1997; Menasche *et al*., 2003). Based on these findings and encouraging experimental evidence, the use of transduced myoblasts has been proposed as a gene delivery system for clinical studies (Moisset *et al*., 2000; Suzuki *et al*., 2001). An important safety-related aspect is the possibility to control the expression of the transgene by being able to determine the expression level *in vitro* prior to implantation or by tetracycline regulation. This autologous cell-based approach would avoid the immune reactions and toxicities currently seen, for example, with adenovirus-mediated gene transfer (St George, 2003). However, recent reports show that retrovirally transduced human cells carry the potential of malignant transformation (Hacein-Bey-Abina *et al*., 2003). The analysis of the retroviral integration sites into the human cells by linear amplification-mediated PCR could make it possible to avoid this adverse effect by analyzing the transduced cells prior to injection (Schmidt *et al*., 2003). It is presently unclear, whether the viral and nonviral gene delivery systems currently used in clinical trials will lead to clinically beneficial therapeutic angiogenesis with an acceptable safety profile (St George, 2003). While these large-scale trials are ongoing, the development of alternative gene delivery systems could be important, including readily controlled and extensively characterized cell-based approaches such as myoblast-mediated gene transfer.

Conclusion

The next steps in angiogenesis research will involve establishing the role and interplay of different angiogenesis inducers and maturation factors. Timing and dosage of these factors alone and in combination need to be studied in order to learn how they can be manipulated to give rise to functional, stable and mature vessels in a controlled way. Myoblast-mediated gene transfer can contribute to this research, as it allows the robust and long-term expression of both 'induction' and 'maturation' factors alone or in combination. The use of regulatable retroviral constructs and myoblast subpopulations could contribute to this effort, making it possible to test the effects of dosage and timing of expression within the same system systematically.

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